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14. ABSTRACT
We have proposed that Akt/mTOR signaling mediates cell survival and contributes to radioresistance. We intend to investigate the cellular and molecular mechanism by which inhibition of Akt/mTOR or overexpression of PTEN in both prostate cancer and its vasculature results in radiosensitization. In addition, we propose to determine possible association between level or activity of these molecules and clinical response to radiotherapy. We have found differences in how irradiation affects Akt/mTOR signaling and in efficacy toward prostate cancer cells when radiation and mTOR inhibitors are combined. We found alternative death mechanisms such as autophagy are important in determining radiation sensitivity of prostate cancer cells. Furthermore, we found inhibition of caspases improves radiation efficacy upon vasculature and prostate cancer cells, by induction of autophagy. We plan to investigate small molecule compounds targeting these pathways for radiosensitization of prostate cancer.

15. SUBJECT TERMS
Prostate cancer, radiotherapy, Akt, Pten, mTOR

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1. Introduction:

We have proposed that Akt/mTOR signaling mediates cell survival and contributes to radioresistance. We intend to investigate the cellular and molecular mechanism by which inhibition of Akt/mTOR or overexpression of PTEN in both prostate cancer and its vasculature results in radiosensitization. In addition, we propose to determine possible association between level or activity of these molecules and clinical response to radiotherapy.

2. Body:

Task 1. To determine whether Akt and mTOR contribute to radiation resistance in prostate cancer and its vascular endothelium (Months 1-12):

a. Determine whether Akt is required for the survival phenotype in irradiated prostate cancer and its vascular endothelium, via adenoviral transfer of dominant-negative Akt (Months 1-4): Our published data showed increased Akt in irradiated prostate cancer cells and irradiated endothelial cells.

b. Determine whether irradiation activates mTOR signaling pathway in prostate cancer cells or endothelial cells, by Western analyses (Months 5-8): Our published data showed increased mTOR signaling in irradiated prostate cancer cells and irradiated endothelial cells.

c. Determine whether inhibition of mTOR by Rapamycin enhances radiotherapy in prostate cancer cells or endothelial cells, by regulating cell survival or apoptosis (Months 9-12): Our published data demonstrated that mTOR inhibitors such as Rapamycin and Rad001 sensitized both prostate cancer cells and endothelial cells to radiation through increased apoptosis and autophagy.

Task 2. To determine whether Akt and mTOR contribute to radiation resistance in prostate cancer:

a. Determine whether Akt is required for the survival phenotype in irradiated prostate cancer: Our published data showed that PTEN-mutated prostate cancer cells had elevated Akt levels and were more resistant to therapy.

b. Determine whether irradiation activates mTOR signaling pathway in prostate cancer cells: Our published data showed that radiation activates mTOR signaling as shown by increased levels of phospho-S6 protein

c. Determine whether inhibition of mTOR by Rapamycin enhances radiotherapy in prostate cancer cells: We found that Rad001 induced autophagy in prostate cancer cells. Inhibition of apoptosis unexpectedly sensitized prostate cancer cells to radiation.

Task 3. To determine the biological significance of Akt/mTOR and PTEN in improving radiation effectiveness in treating prostate cancer. (Months 25-36):

a. Detect changes of tumor size after xenografts (128 nude mice needed) are treated with DN-Akt/PTEN/Rapamycin with or without irradiation. (Months 25-30).

b. Detect changes of tumor blood flow after xenografts (128 nude mice needed) are treated with DN-Akt/PTEN/Rapamycin with or without irradiation, using tumor blood window model or power Doppler Sonography (Months 31-36).

Our original plan was to translate the data in cell culture model to studies using xenografts, which could provide basis for future clinical protocols. Because of our unexpected findings that a pan-caspase inhibitor sensitizes prostate cancer to radiation, we validated the biological aspect of this finding, using caspase 3/7 -

/- MEF cells. We found that radiation-induced autophagy can enhance the cytotoxicity of radiation and is accelerated by inhibiting caspases. This process is mediated by ER stress proteins. We found that synergistic induction of autophagy and radiosensitization when inhibitors of mTOR and caspases are combined.

Since there is no clinically relevant inhibitor of Akt or activator of PTEN, mTOR inhibitors may be the only potential radiosensitizers for patients with prostate cancer. We propose that caspase inhibitors (several of these are currently in clinical studies) are more likely to be used in the clinic to synergize radiosensitization with mTOR inhibitors. We are currently testing Z-VAD and MK867 (a small molecule inhibitor of caspases from Merck) in irradiated prostate cancer xenograft models, with or without mTOR inhibitors.

Increased radiosensitivity of cells with inhibited caspases: We have published recently that inhibition of Bak/Bax results in radiosensitization {Kim, 2006}. To investigate the role of caspases on radiation sensitivity, we used double-knockout (DKO) MEF cells that are deficient for caspase 3/7. These cells have been shown to be unable to undergo apoptosis since they lack caspases 3/7 that execute the apoptotic process. Although inhibition of apoptosis is believed to cause therapeutic resistance, we found the DKO cells more sensitive to radiation, consistent with our published data on Bak/Bax deficient cells {Kim, 2006}. As shown in Figure 1A, the Caspase 3 and 7 -/- MEF cells were more radiosensitive, compared to the WT MEFs (DER=1.65, $P<0.004$). Alternatively, the wild-type (WT) MEF cells were treated with a pan-caspase inhibitor, Z-VAD were also more sensitive to radiation, compared to the vehicle-treated WT MEF cells (DER=1.36, $P<0.003$), as determined by the clonogenic assay. These results suggest that the MEF cells that are deficient or inhibited in caspases are more radiation-sensitive, contrary to our expectations.

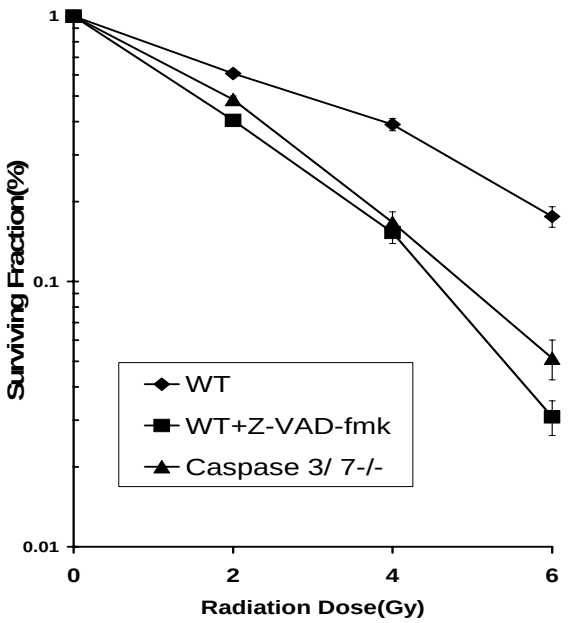
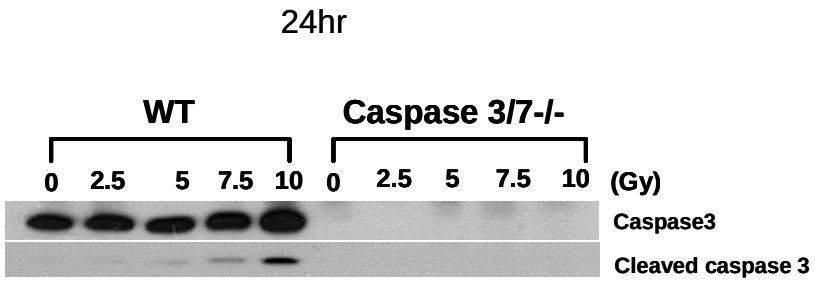


Figure 1. Radiation sensitization when caspases are inhibited: Wild-type (WT) treated with or without Z-VAD and Caspase 3/7 -/-MEF were irradiated with various doses of radiation. After 10 days, surviving colonies were stained and scored. Data presented in the graph is based on three separate repeated experiments and shown as the mean. +/- S.D.

Radiation-induced apoptosis is blocked in the absence of Caspase 3/7: To test whether there is a difference in caspase-dependent apoptosis following irradiation between WT and Caspase 3/7 $^{-/-}$ MEF cells, we examined the cleavage of caspase 3 by Western blot. As shown in Figure 2, proteolytic cleavage of caspase 3 was detected in a dose-dependent manner in WT cells. However, total or cleaved caspase 3 was not detected in the DKO cells as shown in Fig. 2. A corroborative assay, Annexin V-FITC staining was performed to measure percent apoptosis in the irradiated WT and DKO cells (Figure 2). 24 h following 5 Gy, the percentage of apoptotic cells was increased by 6.5-fold in WT cells, and 10 Gy induced an 8.2-fold increase. As expected, in the irradiated Caspase DKO cells there was minimal change in the percentage of apoptotic cells after 5 or 10 Gy, suggesting that these cells undergo cell death independent of apoptotic mechanism. These data suggest that increased radiosensitivity of DKO cells is unlikely caused by ineffective apoptosis in these cells.



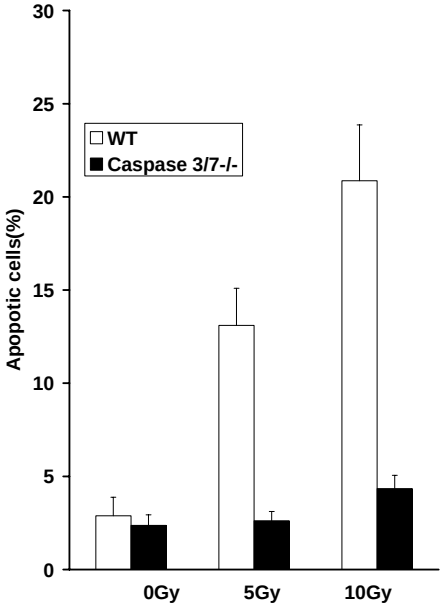
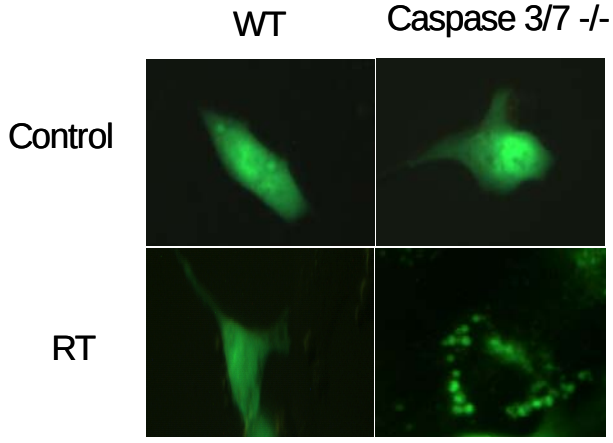


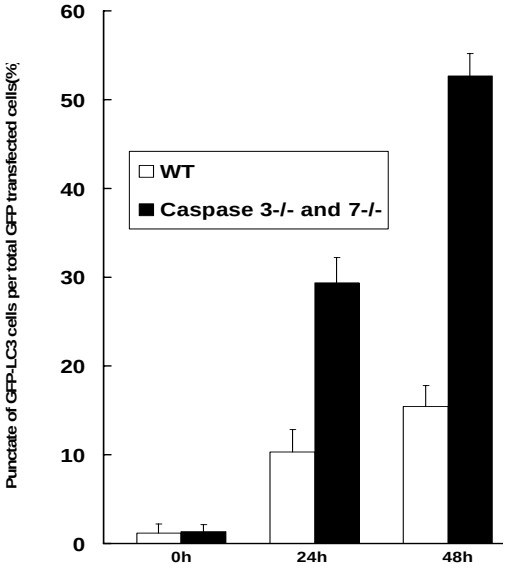
Figure 2. Radiation-induced apoptosis in the presence or the absence of capase 3/7. (A) Caspase-dependent apoptosis was determined by immunoblotting of cleaved caspase 3 after MEF cells were treated with 0, 2.5, 5, 7.5 and 10 Gy. The cells were harvested after 24 hrs. Fifty microgram total proteins per lane were loaded on SDS-PAGE and subjected to Western blot analysis using antibodies. (B) WT and caspase 3/7 -/- MEF cells were incubated with Annexin V-FITC and propidium iodide 24 hr after irradiation, and analyzed by FACScan. Data are shown as the mean of three experiments +/- SD.

Radiation-induced autophagy in the absence of Caspase 3/7: Previously we demonstrated that MEFs deficient in Bax/Bak are radiosensitive. Furthermore increased susceptibility to radiation in Bax/Bak DKO cells primarily stem from increased autophagic cell death {Kim, 2006}. We reasoned that increased radiation sensitivity in Caspase DKO may also be due to autophagy. To address this hypothesis, we performed transfection experiment w/ GFP-LC3 and monitored expression of GFP in both WT and Caspase 3/7 DKO as a marker for autophagy. LC3 have been shown previously to be a specific mammalian marker of autophagosome, a critical component of autophagy {Mizushima, 2001}. After we exposed WT and Caspase DKO cells with 5 Gy of radiation, we observed predominantly punctate pattern of LC3-GFP expression within the cytoplasm in Caspase DKO cell after irradiation, where as WT and control Caspase DKO cells showed diffuse cytoplasmic GFP expression (Fig 3A). The % of punctate cells were increased approximately 3 fold in irradiated Caspase DKO cells, as compared to irradiated WT cells (10% vs. 30%) (Fig, 3B). At 48 hours post radiation, % of punctate cell increased to ~55% in Caspase DKO cells compared to 15% in WT control. These results were also confirmed by accessing the level of LC3 protein expression, in which the Caspase DKO cell showed increase in LC3 protein level following irradiation, as compared to WT cells (Fig. 3C). These results revealed that majority of the cell death in Caspase DKO cells are via autophagy.

A).



B).



C).

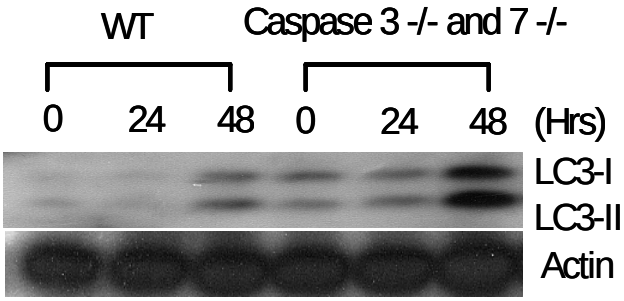


Figure3. Induced autophagy in irradiated Caspase 3/7 deficient cells. A: GFP-LC3 transfected cells were treated with and without 5 Gy and then examined by fluorescence microscopy after 24 hours. The punctate pattern of GFP-LC3 was detected in the irradiated Caspase 3/7 -/-MEF cells. **B:** The percentage of Caspase 3/7 -/- MEF cells with punctate GFP-LC3 fluorescence was calculated relative to all GFP-positive cells. Error bar is shown as mean ± S.D. **C:** LC3 protein processing that is increased during autophagy was detected by western blotting. Actin was probed to demonstrate equal loading.

Autophagy alters radiation sensitivity: Is the autophagy the mechanism responsible for radiosensitivity in Caspase DKO cells? In order to address this question, we knocked down the expression of canonical autophagy signaling molecules in both control and Caspase DKO cells and observed their survival under radiation. For the purposes of this experiment, we utilized two key molecules, ATG-5 and Beclin-1, in autophagy cascade. ATG-5 is one of the 16 autophagy-associated genes (Atg/Apg) and, in conjunction w/ Atg-12, is required to form a complete spherical autophagosome {Mizushima, 2002} {Mizushima, 2003}. Beclin-1 is a mammalian orthologue of yeast Atg6/Vps30 gene and key regulator of autophagy by binding to Bcl-2 {Liang, 1998} {Liang, 1999}. Previously we have shown that siRNA against Beclin-1 and ATG-5 specifically downregulated endogenous protein expression {Kim, 2006}. As mentioned before, Caspase DKO cells show significant reduction on clonogenic survival as compared to wild type at increasing radiation dose. However, when Caspase DKO have been treated with siRNA directed against both ATG-5 and Beclin-1, we observed that the surviving fraction of Caspase DKO cells closely mirrored those of wild type control (DER=1.34, p=0.008) (Fig. 4A), whereas control siRNA treatment of Caspase DKO cells mirrored those of untreated Caspase DKO cells, demonstrating that majority of radiosensitive cell death in Caspase DKO cells is due to autophagy. Conversely, we also found that overexpression of Beclin-1 and Atg-5 in Caspase DKO cells caused significant increase in clonogenic cell death under increasing radiation as compared to Beclin-1 and ATG-5 overexpressing WT cells (DER= 1.32 , p=0.008) (Fig. 4B). Together, these results implicate Caspase DKO cells. Interestingly compared to WT cells overexpressing Beclin-1 and Atg-5, Caspase DKO cells had statistically insignificant differences in clonogenic survival.

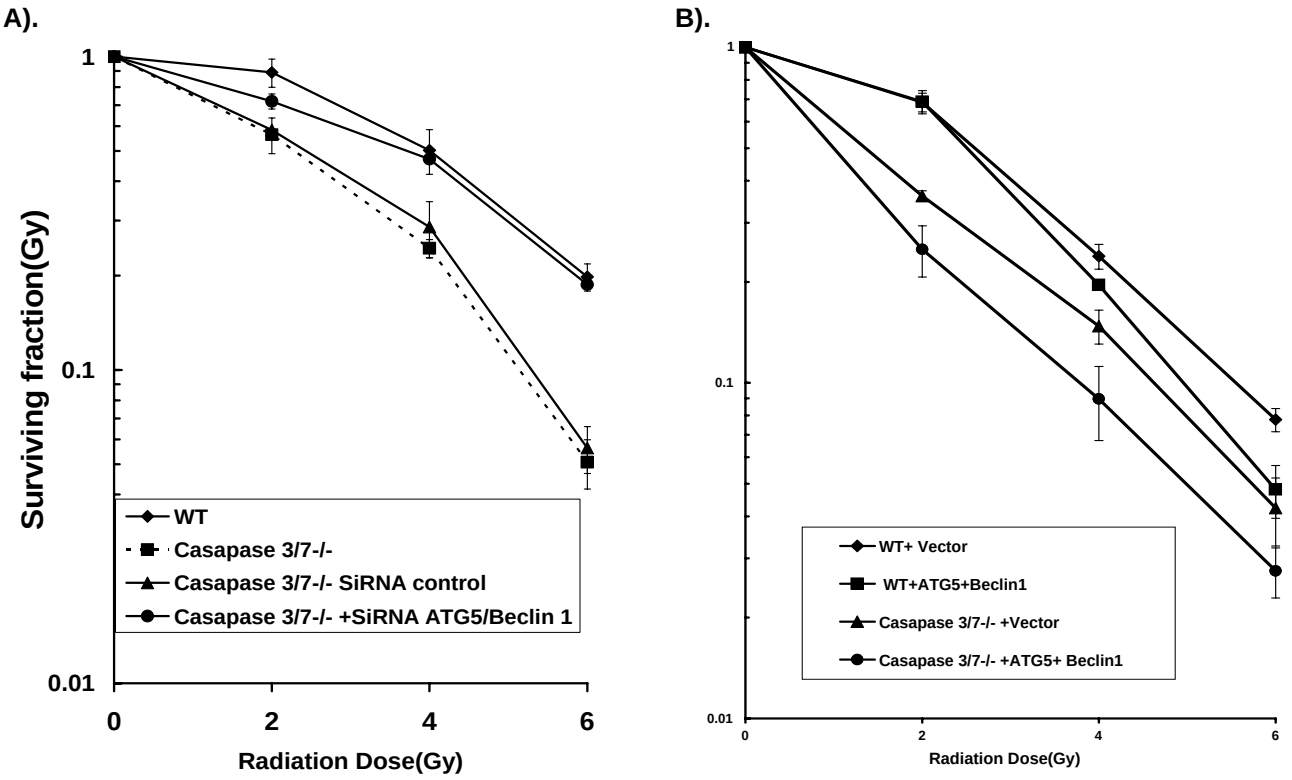
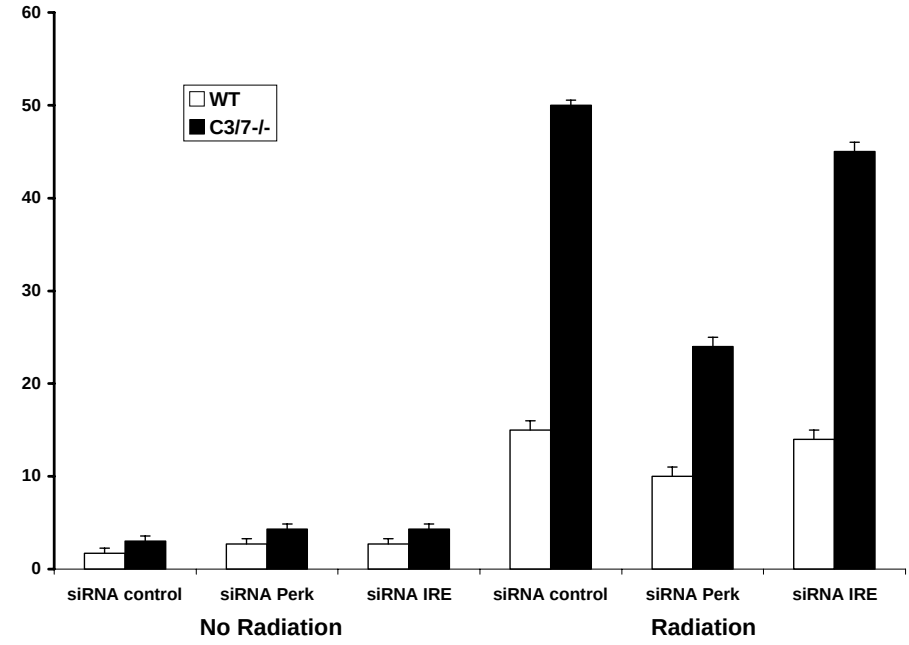


Figure 4. Autophagy alters radiation sensitivity:
A): Wild-type (WT) and caspase 3/7 -/-MEF (DKO) cells were transfected with 25nM siRNA against ATG5 and siRNA against Beclin-1 in combination, or a control siRNA. **B:** WT and DKO cells were transfected with expression plasmids of ATG5 and Beclin-1. They were then irradiated with 0-6Gy. After 8 days, colonies were stained and scored. Data is based upon three separate repeated experiments and shown as the mean +/- S.D.

ER stress signaling mediates radiation-induced autophagy: To understand further how Caspase inactivity results in increased autophagic cell death following radiation, we sought to identify the specific mechanism by which this occurs. Recently it has been suggested that ER stress stimulated the assembly of autophagosomal structure in Atg-dependent manner {Yorimitsu, 2006}. It has been postulated that ER stress causes distinct mediator proteins in the ER membrane for proper response to the stress. One of the pathways involves activation of Protein kinase R-like ER kinase (PERK), which in turn phosphorylates eIF2α to inhibit new protein translation {Marciniak, 2006}. Another pathway involves activation of ER transmembrane stress sensor, IRE1, leading to activation of genes involved in protein degradation. Using siRNA technology, we knocked down the expression of either PERK or IRE1 both in WT and Caspase DKO cells. When we knocked down PERK in Caspase DKO, We observed that radiation caused significant decrease of cell death following radiation (25% cell death), as compared to siRNA control treated Caspase DKO (50% cell death) (Fig 5A). However knocking down IRE1 in Caspase DKO cells resulted in a milder decrease in cell death, compared to siRNA control treated DKO (Fig. 5A). In addition, we observed that radiation treatment caused significant increase in eIF2α phosphorylation in Caspase DKO cells, compared to WT cells as shown in Figure 5B, suggesting that Caspase DKO cell are more susceptible for ER stress. Taken together our data suggest that ER stress is a key component of autophagic cell death, primarily via PERK-eIFα signaling cascade.

A).



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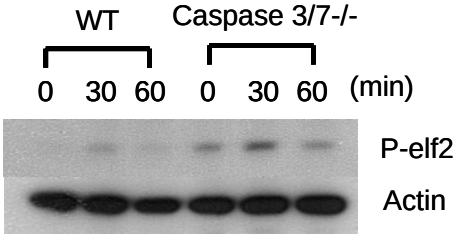

Figure 5. ER stress proteins mediate radiation-induced autophagy: A): Wild-type (WT) and caspase 3/7 -/-MEF (DKO) cells were transfected with 25nM siRNA against PERK, siRNA against IRE, or a control siRNA. GFP-LC3 reporter was co-transfected, which was followed with or without radiation. The percentage of MEF cells with punctate GFP-LC3 fluorescence was calculated relative to all GFP-positive cells. Error bar is shown as mean ± S.D. **B:** WT and DKO cells were irradiated and phospho-elf2 was examined by western blotting.

Establishing clinical database:

We found 98 Caucasian patients with prostate cancer who underwent prostatectomy at Vanderbilt Hospital in 1997. The study was approved by Institutional Review Board (IRB# 030986) at Vanderbilt University School of Medicine. All patients had clinically localized prostate cancer and were treated with radical prostatectomy as a primary treatment. All patients had adenocarcinoma confirmed histologically. The patients were followed at Vanderbilt Hospital or at local hospitals with a mean follow-up of 62 months. Patients` age ranged 40 to 81 with a median age of 63 years. All pathological information was reviewed by one pathologist and the tumor differentiation was evaluated using Gleason`s score criteria. Clinical stage was classified according to the AJCC TNM staging system. The Clinical and histological characteristics of the patients are summarized in Table 1.

TABLE I. *Associations of ATM polymorphism with other clinical and pathologic parameters*

Factor	IVS62+60 Polymorphism (n)			*P* Value
	IVS62+60G/G	IVS62+60A/A	IVS62+60G/A	
Age (yr)				
≤60	7 (20.59)	20 (58.82)	7 (20.59)	
60–70	10 (19.23)	23 (44.23)	19 (36.54)	0.4972
>70	1 (8.33)	7 (58.33)	4 (33.33)	
PSA (ng/mL)				
≤4	5 (29.41)	8 (47.6)	4 (23.53)	
4–10	10 (19.23)	27 (51.92)	15 (28.85)	0.5550
>10	3 (10.34)	15 (51.72)	11 (37.93)	
Gleason score				
≤6	12 (19.35)	33 (53.23)	17 (27.42)	
>6	6 (16.67)	17 (47.22)	13 (36.11)	0.6702
Clinical stage				
I	2 (22.22)	3 (33.33)	4 (44.44)	
II	12 (17.91)	39 (58.21)	16 (23.88)	0.2149
III	4 (18.18)	8 (36.36)	10 (45.45)	

Key: PSA = prostate-specific antigen.
Data in parentheses are percentages.

Tissue preparation and DNA extraction

Using a standard microtome with disposable blades, 5um thickness sections of a representative areas of normal prostatic glands were cut from the paraffin embedded blocks and stained with Hematoxylin and eosin(H&E) and then examined under a microscope to verify the absence of prostate cancer. 5um thickness section (about 1ug) from each patient was used for DNA extraction. The sections were deparaffinized with xylene at room temperature for 30minutes twice. Then the deparaffinized tissue was washed with 100% ethanol twice. After the ethanol had evaporated completely, the tissue was completely lysed with proteinase K. Then QIAamp DNA Mini Kit(QIAGEN Inc, Valencia, CA) was used to extract and purify DNA from the tissues according to the tissue protocol of the kit.

Polymorphism Genotyping

The allelic discrimination of the ATM gene IVS62_60G/A polymorphism was assessed with the ABI Prism 7900 HT Sequence Detection System. Polymerase chain reaction was performed with a total volume of 5 _L, which contained approximately 2.5 ng DNA, 1 _ Taqman Universal PCR Master Mix, each primer at a concentration of 900 nM, and each probe at a concentration of 200 nM. The Taqman probes were as follows: A allele specific, 5=-VIC-TCT TAC CAG GTA GAC TGT GTA TCT CAT CAG GAA GTC ACT GAT GTG AAG AGC-NFQ-3=; and G-allele specific, 5=-FAM- TCT TAC CAG GTA GAC TGT GTA TCT CGT CAG GAA GTC ACT GAT GTG AAG AGC-NFQ-3=. The thermal cycling conditions were as follows: 95°C for 10 minutes to activate the AmpliTaq Gold enzyme, followed by 40 cycles of 15 seconds at 95°C and 60 seconds at 60°C. The fluorescence levels were measured with an ABI PRISM 7900 HT Sequence Detector and resulted in clear identification of three genotypes of each polymorphism. The laboratory staff was unaware of the identity of the men. Quality controls were included in the genotyping assays. Each 384 well plate contained four water, eight CEPH 1347-02 DNA, and eight blinded quality control samples, and unblended quality control samples. The blinded and unblended quality control samples were taken from the second tube of the study samples included in this study.

Statistical analysis

RFS was defined as the time between the date of the primary surgery to the date of relapse or the date of late follow-up. The Kaplan-Meier method was used to compute 5-year survival rates, and the log-rank test was applied to test the difference in survival across different genotypes. The association between the ATM polymorphism and the other clinical and pathologic characteristics was analyzed by Fisher's exact test. $P < 0.05$ was considered statistically significant. All statistical analyses were two-sided.

CORRELATION OF IVS62_60G/A POLYMORPHISM WITH OTHER CLINICAL AND PATHOLOGIC PARAMETERS:

With the Taqman SNP genotyping assay, the concordance of the blinded samples was 100%. Genotypes for IVS62_60G/A were successfully determined in 98 samples. The frequency of homozygous IVS62_60G, heterozygous IVS62_60G/A, and homozygous IVS62_60A/A was 18.37% (18 of 98), 30.61% (30 of 98), and 51.02% (50 of 98), respectively. Using Fisher's exact test, ATM polymorphism had no significant relationship with age ($P = 0.4972$), prostate-specific antigen (PSA) level ($P = 0.5550$), Gleason score ($P = 0.6702$), or clinical stage ($P = 0.2149$; Table I). To increase the power of the study, those who were homozygous and heterozygous for the SNP were combined and compared with the prognostic factors. We found that the IVS62_60G_A also had no significant relationship with age ($P = 0.7614$), PSA level ($P = 0.2395$), Gleason score ($P = 0.7938$), or clinical stage ($P = 0.9188$) in this analysis (Table II).

TABLE II. ***Associations of ATM polymorphism with other clinical and pathologic parameters***

	IVS62+60 Polymorphism (n)		
Factor	IVS62+ 60G/G	IVS62+60A/A+ IVS62+60G/A	*P* Value
Age (y)			
≤60	7 (20.59)	27 (79.41)	
60–70	10 (19.23)	42 (80.77)	0.7614
>70	1 (8.33)	11 (91.67)	
PSA (ng/mL)			
≤4	5 (29.41)	12 (70.59)	
4–10	10 (19.23)	42 (80.77)	0.2395
>10	3 (10.34)	26 (89.66)	
Gleason score			
≤6	12 (19.35)	50 (80.65)	
>6	6 (16.67)	30 (83.33)	0.7938
Clinical stage			
I	2 (22.22)	7 (77.78)	
II	12 (17.91)	55 (82.09)	0.9188
III	4 (18.18)	18 (81.82)	

Key: PSA = prostate-specific antigen.
Data in parentheses are percentages.

FIVE-YEAR RFS

The Kaplan-Meier method was used to compute the survival rates, and the log-rank test was used to test the difference in survival across different genotypes. The 5-year RFS rate for this group was 87.71%. No significant difference was found between those with IVS62_60G/G and those with IVS62_60G/A or IVS62_60A/A in RFS (log-rank test, *P*_0.4533; Table III). The combination of the homozygous and heterozygous subjects for the SNP had little effect on the correlation between this ATM polymorphism and RFS ($P = 0.4470$; Table IV).

TABLE III. *Association of ATM polymorphism with recurrence*

Genotype	Patients (n)	Recurrence (n)	*P* Value
IVS62+60G/G	18	2 (11.11)	
IVS62+60G/A	50	8 (44.44)	0.4533
IVS62+60A/A	30	8 (44.44)	

Data in parentheses are percentages.

TABLE IV. *Association of ATM polymorphism with recurrence*

Genotype	Patients (n)	Recurrence (n)	*P* Value
IVS62+60G/G	18	2 (11.11)	0.4470
IVS62+60G/A+ IVS62+60A/A	80	16 (88.88)	

Data in parentheses are percentages.

3. Key Research Accomplishments:

1). PTEN mutation results in elevated levels of AKT and radiation resistance.
2). Radiation activates Akt/mTOR signaling.
3). mTOR inhibitors induce autophagy and sensitize prostate cancer cells to radiation.
4). Caspase inhibitors enhances radiation-induced autophagy through activation of ER stress and synergize with mTOR inhibitors for radiosensitization.

4. Reportable outcomes:

1. Cao C, Subhawong T, Albert JM, Kim KW, Geng L, Sekhar KR, Gi YJ, **Lu B**. Inhibition of mammalian target of rapamycin or apoptotic pathway induces autophagy and radiosensitizes PTEN null prostate cancer cells. *Cancer Res. 2006 Oct 15;66(20):10040-7.*

2. Kwang Woon Kim, Robert W. Mutter, Carolyn Cao, Jeffrey M. Albert, Michael Freeman, Dennis E. Hallahan, and **Bo Lu.** Autophagy for cancer therapy through inhibition of proapoptotic proteins and mTOR signaling. *J Biol Chem. 2006 Dec 1;281(48):36883-90.*

3. Eric Shinohara, Kenneth Niermann, Carolyn Cao, Fenghua Zeng, Dennis E. Hallahan, and Bo Lu. mTOR inhibitors as a potential anti-angiogenesis agent enhanced efficacy of radiotherapy. *Oncogene 2005 24: 5414-5422.*

4. Robert Lee Browning, Hecheng Li, Eric T Shinohara, Qiuyin Cai, Heidi Chen, Regina Courtney, Carolyn Cao, Wei Zheng, and **Bo Lu** ATM polymorphism IVS62+60G>A is not associated with aggressiveness of disease in prostate cancer. *Urology. 2006 Jun;67(6):1320-3.*

5. Li HC, Eric Shinohara and Bo Lu. Endostatin polymorphism 4349G/A(D104N) is not Associated with Aggressiveness of Disease in Prostate Cancer *Dis Markers* Volume 21, Number 1, 2005

6. Li HC, Eric T Shinohara and Bo Lu Plasminogen activator inhibitor-1(PAI-1) promoter polymorphism is not associated with prognosis in prostate cancer. *Clinical Oncology, Volume 18, Issue 4, May 2006, Pages 333-337.*

5. Recent Publications:

1). Luigi Moretti, Kwang Woon Kim, and **Bo Lu**. Crosstalk between Bak/Bax and mTOR signaling regulates radiation-induced Autophagy. Autophagy 2007 Apr-Jun;3(2):142-4.

2). Luigi Moretti, Yong Cha, Kenneth Niermann, and **Bo Lu**. Switch between Apoptosis and Autophagy: Radiation induced-Endoplasmic Reticulum Stress? Cell Cycle 6(7), 2007 April 1.

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